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2.	Patent application number *(The Patent Office will fill in this part)*	0327907.2 - 2 DEC 2003
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Ciba Specialty Chemicals Water Treatments Limited Cleckheaton Road Low Moor Bradford West Yorkshire BD12 0JZ
	Patent ADP number *(if you know it)*	7585391005
	If the applicant is a corporate body, give the country/state of its incorporation	England
4.	Title of invention	MICROORGANISM
5.	Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Ciba Specialty Chemicals Water Treatments Limited Patents Department PO Box 38 Cleckheaton Road Low Moor Bradford West Yorkshire BD12 0JZ
	Patents ADP number *(if you know it)*	07585391002

		Country	Priority application number *(if you know it)*	Date of filing (day/month/year)
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8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *(see note (d))*	YES

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11. I/We request the grant of a patent on the basis of this application

Signature: [signature] Date: 02 December 2003

12. Name and daytime telephone number of person to contact in the United Kingdom: Catherine Choppen 01274 417446

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Microorganism

The present invention relates to a microorganism and to methods of culturing the microorganism. The invention also relates to a novel nitrile hydratase enzyme and also to a method of converting a nitrile to an amide employing the nitrile hydratase enzyme.

It is well known to employ biocatalysts, such as microorganisms that contain enzymes, for conducting chemical reactions. Nitrile hydratase enzymes are known to catalyse the hydration of nitriles directly to the corresponding amides. Typically nitrile hydratase enzymes can be produced by a variety of microorganisms, for instance microorganisms of the genus Bacillus, Bacteridium, Micrococuss, Brevibacterium, Corynebacterium, Pseudomonas, Acinetobacter, Xanthobacter, Streptomyces, Rhizobium, Klebsiella, Enterobacter, Erwinia, Aeromonas, Citrobacter, Achromobacter, Agrobacterium, Pseudonocardia and Rhodococcus.

Many references have described the synthesis of nitrile hydratase within microorganisms. Arnaud et al., Agric. Biol. Chem. 41: (11) 2183-2191 (1977) describes the characteristics of an enzyme they refer to as 'acetonitrilase' in Brevibacterium sp R312 which degrades acetonitrile to acetate via the amide intermediate. Asano et al., Agric. Biol. Chem. 46: (5) 1183-1189 (1982) isolated Pseudomonas chlororaphis B23 which produced nitrile hydratase to catalyse the conversion of acrylonitrile to acrylamide, generating 400 g/L acrylamide. The article by Yamada et al., Agric. Biol. Chem. 50: (11) 2859-2865 (1986) entitled, "Optimum culture conditions for production by Pseudomonas chlororaphis B23 of nitrile hydratase", considered the optimisation of the medium components of the growth medium, including the inducer added for nitrile hydratase synthesis. Methacrylamide was found to be the best inducer for this organism. Methacrylamide was included in the culture at the start of growth.

Various strains of the Rhodococcus rhodochrous species have been found to very effectively produce nitrile hydratase enzyme.

EP-0 307 926 describes the culturing of Rhodococcus rhodochrous, specifically strain J1 in a culture medium that contains cobalt ions. A process is described for biologically producing an amide in which a nitrile is hydrated by the action of a nitrile hydratase produced by Rhodococcus rhodochrous J1, which has been cultured in the presence of cobalt ion. The use of various inducers (including crotonamide) is described for the synthesis of nitrile hydratase. In one embodiment an amide is produced in a culture medium of the microorganism in which a substrate nitrile is present. In another embodiment a substrate nitrile is added to the culture medium in which a nitrile hydratase has been accumulated to conduct the hydration reaction. There is also a description of isolating the microorganism cells and supporting them in a suitable carrier, for instance by immobilisation, and then contacting them with a substrate. The nitrile hydratase can be used to hydrate nitriles into amides, and in particular the conversion of 3-cyanopyridine to nicotinamide.

EP-0 362 829 describes a method for cultivating bacteria of the species Rhodococcus rhodochrous comprising at least one of urea and cobalt ion for preparing the cells of Rhodococcus rhodochrous having nitrile hydratase activity. Specifically described is the induction of nitrile hydratase in Rhodococcus rhodochrous J1 using urea or urea derivatives which markedly increases the nitrile hydratase activity. Urea or its derivatives are added to the culture medium in one batch at a time or sequentially and cultivation occurs over 30 hours or longer, for instance up to 120 hours.

An article by Nagasawa et al., Appl. Microbiol. Biotechnol. 34: 783-788 (1991), entitled "Optimum culture conditions for the production of cobalt-containing nitrile hydratase by Rhodococcus rhodochrous J1", describes isolating J1 as an

acetonitrile utilising strain which synthesises two different nitrile hydratases and a nitrilase depending upon the culture conditions used. One nitrile hydratase is induced optimally by urea and urea analogues. Urea is added at the start of the culturing process and seems to become efficient as an inducer only when the basal medium is nutrient rich. Induction of the enzyme started gradually and increased in growth until it reached a maximum after 5 days of cultivation. The activity was found to decrease on prolonged cultivation.

Rhodococcus rhodochrous J1, is also used commercially to manufacture acrylamide monomer from acrylonitrile and this process has been described by Nagasawa and Yamada Pure Appl. Chem. 67: 1241-1256 (1995).

Leonova et al., Appl. Biochem. Biotechnol. 88: 231-241 (2000) entitled, "Nitrile Hydratase of Rhodococcus", describes the growth and synthesis of nitrile hydratase in Rhodococcus rhodochrous M8. The nitrile hydratase synthesis of this strain is induced by urea in the medium, the urea also acting as a nitrogen source for growth by this organism. Cobalt is also required for high nitrile hydratase activity. This literature paper looks at induction and metabolic effects in the main.

Leonova et al., Appl. Biochem. Biotechnol. 88: 231-241 (2000) also states that acrylamide is produced commercially in Russia using Rhodococcus rhodochrous M8. Russian patent 1731814 describes Rhodococcus rhodochrous strain M8.

Rhodococcus rhodochrous strain M33 that produces nitrile hydratase without the need of an inducer such as urea is described in US-A-5827699. This strain of microorganism is a derivative of Rhodococcus rhodochrous M8.

The production of acrylamide monomer in particular is desirable via the biocatalytic route. In the review publication by Yamada and Kobayashi, Biosci.

Biotech. Biochem. 60: (9) 1391-1400 (1996) titled "Nitrile Hydratase and its Application to Industrial Production of Acrylamide" a detailed account of the development of a biocatalytic route to acrylamide is described. Three successively better catalysts and their characteristics for acrylamide production and in particular the third generation catalyst Rhodococcus rhodochrous J1 are described in some detail.

WO-A-02/088373 describes a method and device for producing an aqueous acrylamide solution. A recycling loop containing a heat exchanger is used to remove heat of reaction and also to control the dosing of acrylonitrile.

WO-A-02/088371 describes a method and device for producing an aqueous acrylamide solution in which FTIR is used to monitor and control the reaction.

WO-A-02/50297 describes a process for producing an amide compound, one example of an amide being acrylamide using a microbial catalyst in which the catalyst is not entrapped. Therefore the free cell process can be carried out as a batch or continuous reaction.

A disadvantage with the prior art processes and biocatalysts is that normally a biocatalyst for preparing amides must be immobilised in some way, for instance entrapped in a polymer matrix, if it is to be used for any period time or only used to make one batch of amide. Therefore in the absence of immobilisation the longevity of the microorganism and enzyme tends to be relatively short. In particular once a biocatalyst has been used in a process to generate an amide by hydration of the corresponding nitrile, the biocatalyst has normally lost a significant amount of its activity and therefore would not be considered suitable for recycling and reuse in subsequent reactions.

In addition the length of time to culture a microorganism that synthesises nitrile hydratase to a satisfactory level of activity is undesirably long when urea is used

as the nitrile hydratase inducer.

A further problem is that microorganisms that produce nitrile hydratase enzyme also simultaneously produce amidase with the result that a proportion of the amide that is formed during the reaction is subsequently converted to the corresponding carboxylic acid. This has the disadvantages that the yield of amide produced is diminished and the possible deleterious effects of quantities of carboxylic acid in the amide product. This is particularly a problem in the manufacture of acrylamide where the intention is to form acrylamide homopolymer or more significantly cationic polyacrylamides where the presence of significant amounts of anionic acrylic acid monomer may bring about insolubilisation and/or effective loss of net cationic content.

It would therefore be desirable to provide a process and a biocatalyst where these disadvantages can be overcome.

According to the present invention we provide a microorganism that is Rhodococcus rhodochrous strain 2368 (NCIMB 41164) or a mutant thereof.

This new microorganism has been found to readily produce nitrile hydratase. We have found that this new microorganism (and the nitrile hydratase produced therefrom) can be used in a process of converting nitriles, to the amide. Rhodococcus rhodochrous 2368 is particularly of use for the conversion of (meth)acrylonitrile to (meth)acrylamide. The microorganism and enzyme have been found to remain active, and in some cases increase in activity, over long periods of time and furthermore can easily be recycled and reused without any evident significant loss in activity. Furthermore, we have found that it is possible to suppress the formation of the corresponding amidase, with the effect that no or reduced levels of the corresponding carboxylic acid will be formed.

The details of the new strain Rhodococcus rhodochrous 2368 are given below:

1. Origin and Deposition

The strain 2368 was isolated by us from soil in Bradford, England and deposited on 5th March 2003 at the National Collection of Industrial and Marine Bacteria (NCIMB), where it was assigned the accession number NCIMB 41164 under the Budapest Treaty.

2. Morphological and cultural characteristics

(1) Polymorphic growth

(2) Motility: immotile

(3) Non-spore former

(4) Gram positive

(5) Aerobic

(6) Growth on nutrient agar gives salmon pink round colonies within 48 hours at 30°C

3. Cultivation and Nitrile Hydratase Synthesis

The Rhodococcus rhodochrous 2368 of the present invention can be cultured under any conditions suitable for the purpose in accordance with any of the known methods, for instance as described in the aforementioned prior art. Preferably the microorganism is cultured in a culture medium that comprises urea or a derivative of urea. We have found that this microorganism can be grown in a medium containing acetonitrile or acrylonitrile as an inducer of the nitrile hydratase. In the presence of urea or urea derivative as an inducer and cobalt chloride as a source of cobalt ions, very high nitrile hydratase activity is achieved. For example urea and cobalt are added to the medium described in the experimental examples.

Desirably the biocatalyst can be cultured to give high enzyme activity, for instance about 250-300,000 μmol min^{-1}/g dry biomass at 15°C. High nitrile hydratase activity can preferably be achieved if urea or urea derivative is not present in the culture medium at the start of the microorganism growth but is

introduced later. More preferably the culture medium is substantially free of urea or urea derivative for at least the first six hours of microorganism growth. It is especially preferred if the growth medium of the microorganism is free of urea or urea derivative for at least 24 hours before the introduction of the urea or urea derivative as the growth rate of the microorganism is higher in the absence of urea, but that it is added before 48 hours culturing of the microorganism.

We have found that this enables higher nitrile hydratase activity to occur in a shorter period of time than if the urea had been added at the start of culturing.

The invention also relates to a nitrile hydratase obtainable from a microorganism which is Rhodococcus rhodochrous 2368 or a mutant thereof.

A further aspect of the invention concerns a process of preparing an amide from the corresponding nitrile wherein the nitrile is subjected to a hydration reaction in an aqueous medium in the presence of a biocatalyst selected from the group consisting of a microorganism which is a Rhodococcus rhodochrous 2368, a mutant thereof and a nitrile hydratase obtainable from Rhodococcus rhodochrous 2368 or a mutant thereof.

This process is particularly suitable for readily preparing an amide from the corresponding nitrile. In particular aqueous solutions of amide can be prepared in high concentration. The process is especially suitable for preparing acrylamide or methacrylamide.

The microorganism may be used as a whole cell catalyst for the generation of amide from nitrile. It may be immobilised for instance entrapped in a gel or it may be used preferably as a free cell suspension. Alternatively the nitrile hydratase enzyme may be extracted and for instance used directly in the process of preparing the amide.

In one preferred way of carrying out the process the biocatalyst is introduced into an aqueous medium suitable for carrying out the culturing of the microorganism. Typically a suspension of the biocatalyst, for instance whole cells of the microorganism, may be formed. A nitrile, for instance acrylonitrile or methacrylonitrile is fed into the aqueous medium comprising the biocatalyst in such a way that the concentration of (meth) acrylonitrile in the aqueous medium is maintained at up to 6% by weight. Nitrile such as acrylonitrile or methacrylonitrile is more preferably fed into the reaction medium and the reaction allowed to continue until the concentration of amide, for instance acrylamide or methacrylamide reaches the desired level, in particular between 30 and 55% by weight. Most preferably the concentration is around 50% by weight.

This new strain of Rhodococcus rhodochrous (2368) is capable of producing aqueous acrylamide solutions in high concentration (for instance 50% acrylamide). Desirably reaction may be carried out as a free cell process using a fed-batch type reactor to which biocatalyst is added in the form of fermentation broth or as harvested biomass.

The activity of the biocatalyst microorganism Rhodococcus rhodochrous 2368 and the nitrile hydratase produced therefrom is such that it can be recycled and reused for further hydration of nitrile to the corresponding amide.

We have found that in the process of the invention for preparing an amide such as acrylamide, the biocatalyst (for instance as free cells of the microorganism) can be recycled and reused at least once without any loss in enzyme activity. Furthermore the amount of carboxylic acid formed as a by-product (as a result of amidase) is greatly reduced when the recycled catalyst is used. Without being limited theory it is thought that in the amidase enzyme may be inhibited by the high acrylamide concentrations.

Recycling of the biocatalyst is particularly suitable for any case of converting (meth) acrylonitrile to (meth) acrylamide. Thus in the manufacture of acrylamide when the reaction process is complete and acrylamide has been produced at the appropriate concentration, the catalyst can be removed and re-used to produce another batch of acrylamide without loss in nitrile hydratase activity. This can even be achieved after the biocatalyst has been stored in water for several days (for instance three days) prior to reuse. It is even possible to prepare a third batch of acrylamide, even after further storage.

In a further aspect of the invention we have found a way of improving the biocatalytic activity of a microorganism. The microorganism would be cultured in a culture medium that comprises urea or a derivative of urea. However, the culture medium is substantially free of urea or urea derivative for at least the first six hours of culturing the microorganism and thereafter urea or a urea derivative is added to the culture medium. Preferably the culture medium is substantially free of urea or urea derivative for at least 24 hours. However, in order to maximise the biocatalytic activity it is preferred to introduce the urea or urea derivative within 48 hours of culturing.

The biocatalytic activity can be established in terms of enzyme activity as described herein.

Preferably the microorganism is capable of producing a nitrile hydratase. Suitably a biocatalyst comprising such a microorganism can be used to prepare amides from the corresponding nitrile by a hydration process in which nitrile hydratase catalyses the reaction. The culturing of the microorganism by delayed introduction of urea or urea derivative provides increased nitrile hydratase activity particularly suitable for this reaction. The process is particularly suitable for the preparation of (meth) acrylamide from (meth) acrylonitrile. Such a process may be carried out as described herein. In addition the biocatalyst may be recycled and reused.

It is particularly desirable that the microorganism is of the Rhodococcus genus, preferably a Rhodococcus rhodochrous species.

The following examples provide an illustration of how to carry out the invention.

Examples

(1) Rhodococcus rhodochrous 2368 was isolated from soil using an enrichment culture technique and it was grown on a medium containing the following constituents (g/l): KH_2PO_4, 7.0; KH_2PO_4, 3.0; peptone, 5.0; yeast extract, 3.0; glucose, 5.0; $MgSO_4$, 0.5; trace metals solution, 5 ml; acetonitrile, 20 ml. The pH was adjusted to 7.2. The nitrile hydratase activity was 4,000 μmol min^{-1}/g dry cells at 15°C after 3 days growth at 28°C.

(2) Rhodococcus rhodochrous 2368 was grown in a 2L baffled Erlenmeyer flask containing 400 mL culture medium containing the following constituents (g/L): diPotassium hydrogen phosphate 0.7; Potassium hydrogen phosphate 0.3; glucose 10.0; peptone, 1.0; yeast extract 3.0; magnesium sulphate heptahydrate 0.5; Urea 5.0; cobalt chloride hexahydrate 0.01; tap water to 1L. The pH of the medium was adjusted to pH 7.2. The culture was grown at 28°C for 5 days after which the nitrile hydratase activity was 264,000 μmol min^{-1}/g at 15°C.

(3) (a) Rhodococcus rhodochrous 2368 was grown in the medium described in (2) except that peptone was omitted.

Rhodococcus rhodochrous 2368 was grown in the medium described in (3a) except that peptone was omitted as was urea. The organism was cultured for 24 hours and then 5 g/L urea was added to the culture which was grown for a further 5 days.

Rhodococcus rhodochrous 2368 was grown in the medium described in (3a) except that urea was not included in the medium. The organism was cultured for 48 hours and then 5 g/L urea was added to the culture which was grown for a further 4 days.

Rhodococcus rhodochrous 2368 was grown in the medium described in (3a) except that urea was not included in the medium. The organism was cultured for 6 days.

Samples were taken from the four cultures described above at time = 1, 2, 3 and 6 days after growth commenced. The nitrile hydratase activities were measured at 15°C, see table 1.

Table 1

Urea addition time (days)	Nitrile Hydratase Activity µmol min^{-1}/mg			
	T = 1 day	T = 2 days	T = 3 days	T = 6 days
0	9.1	24.2	24.8	37.6
1	1.0	21.6	49.3	41.3
2	ND	ND	15.1	15.3
None added	0.94	ND	0.46	0.98

ND not determined

(4) Rhodococcus rhodochrous 2368 was grown in a 280L fermenter containing 180 L culture medium containing the following constituents (g/L): diPotassium hydrogen phosphate 0.7; Potassium hydrogen phosphate 0.3; glucose 2.0; yeast extract 3.0; magnesium sulphate heptahydrate 0.5; cobalt chloride hexahydrate 0.01;. The pH of the medium was adjusted to pH 7.2. The culture was grown at 30°C for 3 days. Urea was added to the culture when the A600 was ~1.9. The nitrile hydratase activity was measured (at 30°C) periodically.

22 h after the urea was added the activity was approximately 176,000 µmol min^{-1}/g at 30°C and after a further 9 h the activity had increased to 323, 000 µmol min^{-1}/g.

(5) 625 g of water was charged to the reactor to which Rhodococcus rhodochrous 2368 was added. The mixture was heated to 25oC. Acrylonitrile 375 g was fed to the reactor at a rate to maintain the concentration at 2% (w/w). After 175 minutes all of the acrylonitrile had been converted to acrylamide to a final concentration of approximately 50% (w/w).

(6) The cells from 5 were recovered by centrifugation and they were suspended in 625 g water. This suspension was stored at 4oC for 3 days prior to re-charging to the reactor. The procedure described in 5 was followed and again after 175 minutes all of the acrylonitrile was converted to acrylamide.

(7) The cells from 6 were treated as described in 6 above except they were stored for 2 days prior to re-use. Again 50% acrylamide was synthesised, although it took 800 minutes.

(8) Rhodococcus rhodochrous 2368 was added to a reactor containing 150 kg of water. 85 kg of acrylonitrile was fed to the reactor at a rate of 35 kg/h at 25oC. The biocatalyst was recovered by centrifugation.

(9) The cells from 8 were charged back to the reactor with a further 125 kg water. 50% acrylamide was achieved in 170 min with the first reaction and in 145 min with the recycled catalyst.

The acrylic acid concentrations measured for the batches of acrylamide generated in examples 5-9 are shown in Table 1.

Table 1: Acrylic acid concentrations measured in each of the acrylamide batches

Example number	Acrylic Acid Concentration (ppm)
5	5650
6	102
7	None detected (<10 ppm)
8	8500
9	1300

Claims

1. A microorganism which is Rhodococcus rhodochrous strain 2368 (NCIMB 41164) or a mutant thereof.

2. A method of culturing the microorganism Rhodococcus rhodochrous strain 2368 (NCIMB 41164) or mutant thereof in a culture medium comprising urea or a derivative of urea.

3. A method according to claim 2 in which the culture medium is substantially free of urea or urea derivative for at least the first six hours of culturing the microorganism and thereafter urea or a urea derivative is added to the culture medium.

4. A method according to claim 2 or claim 3 in which the culture medium is substantially free of urea or urea derivative for at least the first 24 hours of culturing the microorganism and thereafter urea or urea derivative is added to the culture medium.

5. A method according to any of claims 2 to 4 in which urea or urea derivative is added to the culture medium within 48 hours of culturing.

6. A nitrile hydratase obtainable from a microorganism which is Rhodococcus rhodochrous strain 2368 (NCIMB 41164) or a mutant thereof.

7. A process of preparing an amide from the corresponding nitrile wherein the nitrile is subjected to a hydration reaction in an aqueous medium in the presence of a biocatalyst selected from the group consisting of a microorganism which is a Rhodococcus rhodochrous strain 2368 (NCIMB 41164), a mutant thereof and a nitrile hydratase obtainable from Rhodococcus rhodochrous strain 2368 or a mutant thereof.

8. A process according to claim 7 in which the amide is (meth)acrylamide.

9. A process according to claim 8 in which the biocatalyst is introduced into an aqueous medium and (meth)acrylonitrile is fed into the aqueous medium such that the concentration of (meth)acrylonitrile in the aqueous medium is maintained at up to 6% by weight.

10. A process according to claim 9 in which the reaction continues until the concentration of acrylamide is between 30 and 55% by weight.

11. A process according to any of claims 7 to 10 in which the biocatalyst is recycled and reused.

12. A method of improving the biocatalytic activity of a microorganism, in which the microorganism is cultured in a culture medium that comprises urea or a derivative of urea,
wherein the culture medium is substantially free of urea or urea derivative for at least the first six hours of culturing the microorganism and thereafter urea or a urea derivative is added to the culture medium.

13. A method according to claim 12 in which the culture medium is substantially free of urea or urea derivative for at least the first 24 hours of culturing the microorganism and thereafter urea or urea derivative is added to the culture medium.

14. A method according to claim 12 or claim 13 in which urea or urea derivative is added to the culture medium within 48 hours of culturing.

15. A method according to any of claims 12 to 14 in which the microorganism is capable of producing a nitrile hydratase.

16. A method according to any of claims 12 to 15 in which the microorganism is of the Rhodococcus genus, preferably Rhodococcus rhodochrous species.

17. A process of preparing an amide from the corresponding nitrile wherein the nitrile is subjected to a hydration reaction in an aqueous medium in the presence of a biocatalyst selected from the group consisting of a microorganism which is capable of producing a nitrile hydratase,
wherein the microorganism has been cultured by the method according to any of claims 12 to 16.

18. A process according to claim 17 in which the amide is (meth) acrylamide.

19. A process according to claim 18 in which the biocatalyst is introduced into an aqueous medium and (meth)acrylonitrile is fed into the aqueous medium such that the concentration of (meth)acrylonitrile in the aqueous medium is maintained at up to 6% by weight.

20. A process according to claim 19 in which the reaction continues until the concentration of acrylamide is between 30 and 55% by weight.

21. A process according to any of claims 17 to 20 in which the biocatalyst is recycled and reused.

Abstract

Microorganism

A microorganism which is Rhodococcus rhodochrous strain 2368 (NCIMB 41164) or a mutant thereof. A method of culturing the microorganism in a culture medium comprising urea or urea derivative is claimed. A nitrile hydratase obtainable from the microorganism is claimed. Also claimed is a process of preparing an amide from the corresponding nitrile wherein the nitrile is subjected to a hydration reaction in an aqueous medium in the presence of a biocatalyst selected from the group consisting of a microorganism which is a Rhodococcus rhodochrous strain 2368, a mutant thereof and a nitrile hydratase obtainable from Rhodococcus rhodochrous strain 2368 or a mutant thereof.

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